REMARKS

With entry of this Amendment, claims 1-11 are pending. Applicants thank the Examiner for the telephone discussion of June 4, 2008, providing assistance with the language of claim 1. Applicants have amended claims 1, 3, and 10 to further describe the claimed invention. The specification supports this amendment at, for example, page 2, line 20 to page 3, line 3 and in the Example starting at page 7. These claim amendments therefore do not introduce new matter.

Applicants acknowledge with appreciation the Office's withdrawal of the prior objection to the drawings and the prior double patenting rejection. Applicants address the maintained obviousness rejections below.

Rejections Under 35 U.S.C. § 103

Claims 1-3, 7-9 and 11 remain rejected under 35 U.S.C. § 103(a) as allegedly obvious over JP 5-56990 A ("Kawatani") in view of U.S. Patent 5,609,633 ("Kokubo"). Office Action, page 2. According to the Office, Kawatani teaches a "porous body comprising a lump of titanium or titanium alloy . . . and having a porosity of 40-60%. The body has a larger pore interconnected in a three-dimensional network with a diameter of 100 to 200 μm and smaller holes with diameters of 50 μm or [less]." *Id.* The Office also contends that the "porous network penetrates the rough film/body from one end to the other." *Id.* Considering the varied diameters of the pores in Kawatani, the Office reasons, it is "inevitable" that the claimed structure of a hole on an inner surface of the interconnected pore would appear. *Id.* The Office acknowledges that Kawatani does not teach a film comprising at least one phase selected from the group consisting of an amorphous titanium oxide phase, an amorphous alkali titanate phase, an anatase phase and a rutile phase aligned with (101) plane. *Id.*

The Office contends that Kokubo teaches a film comprising at least one phase selected from the group consisting of an amorphous titanium oxide phase, an amorphous alkali titanate phase, an anatase phase and a rutile phase aligned with (101) plane at column 2, lines 4-13. *Id.* at 3. Kokubo also allegedly teaches that the desirable thickness of the film is 0.1 to 10 μm and that the film is made by immersing the titanium in an alkaline aqueous solution followed by a heat treatment. *Id.*

Taking each of these alleged teachings, the Office concludes that it would have been obvious to combine the film/layer allegedly taught by Kokubo with the porous endosseous implant of Kawatani. *Id.* According to the Office, one would have been motivated to do so because Kokubo suggests that a substrate covered by a film including a phase of alkali titanate would induce the growth of apatite and thus increase the ability of the implant to bond with bones of the body. *Id.*

In the prior response, Applicants explained that the invention in Kawatani focuses on generating a "rough film" with "high porosity" on the *surface* of a "base material." In contrast, the invention of claim 1 has a porous body "comprising a lump of titanium or titanium alloy and having a porosity of 30 to 80%, the body having a pore interconnected in a three-dimensional network with a diameter of 100 to 3000 μm and a hole with a diameter of 50 μm or less on an inner surface of the pore, the pore penetrating from one end of the body to the other end." In Kawatani, the rough film sits on the surface of a base material. Any "pores" that may be present in this rough film clearly do not extend through the base material to the other side because this rough film merely sits on its surface. Thus, Kawatani does not teach an "osteoinductive artificial bone" in which the pore penetrates from one end of the body to the other end.

Combining Kawatani with Kokubo does not remedy the shortcoming of Kawatani because Kokubo teaches only the bioactivity of apatite formed on a titanium plate to enhance bonding of bone to the plate. Kokubo does not discuss osteoinductivity nor does Kokubo teach a "porous body" in which the pore penetrates from one end of the porous body to the other end, conferring osteoinductivity to the artificial bone.

In response to Applicants' argument, the Office now contends that the feature of "pores that penetrate the entire implant" was not recited in the rejected claims. Office Action, page 6. Moreover, the Office agrees with Applicant's description of Kawatani, but dismisses it as irrelevant because, in the Office's opinion, the feature was not in the claims. *Id.*

While Applicants believe that the prior amendment to claims 1, 3, and 10 incorporated this element, Applicants have again amended these claims to clarify that Applicants consider this feature as part of the claimed invention. Specifically, as discussed with the Examiner, Applicants have amended claim 1 to recite an "osteoinductive artificial bone that is a porous body . . . the body having a pore interconnected in a three-dimensional network . . . the pore penetrating from one end of the body to the other end." In addition, claims 3 and 10 have been amended to refer to the osteoinductive artificial bone of claim 1. As Applicants have argued and as the Office agrees, Kawatani does not teach the claimed artificial bone. Likewise, Kokubo does not teach this structure. Therefore, the combination of these two references cannot render obvious the osteoinductive artificial bone of claims 1, 2, and 11 or methods of making the osteoinductive artificial bone of claim 1 as described in claims 3 and 7-9.

Applicants also previously noted that Kawatani's implant requires the presence of a base material to which the rough film is attached to facilitate attachment of the base material to the body via the rough film in a process called "bioactivity." In contrast, the claimed artificial bone has "osteoinductivity," which is the ability to facilitate bone formation even in a body location where bone does not normally form. Even if one could form the film recited in claim 1 on the "rough film" of Kawatani the coated "rough film" would not be osteoinductive as recited in claim 1 because bone formation via osteoinduction begins not at the periphery of an implant, but at the center of the implant. See Amendment filed November 29, 2007, at pages 10 and 11.

The Office now responds by suggesting that the combination of Kawatani and Kokubo would inherently be osteoinductive. Office Action, page 6. In alleged support of this conclusion, the Office relies on the specification at page 2, lines 4-10, which states that "it has been known that a specific ceramic porous body comprising hydroxyl apatite etc. has osteoinductivity, which can induce bone formation in a location in which a bone does not intrinsically exist." *Id.* Based upon this teaching, the Office incorrectly assumes that the reason why the ceramic has osteoinductive properties is because of the layer of apatite formed on its surface. *Id.* The Office also asserts that "the film taught by Kokubo has the ability to 'bond with bones' because a layer of apatite is formed on its surface." *Id.* Based on these premises, the Office concludes that the combination of Kawatani and Kokubo "appears to have the necessary properties (porosity and a layer of apatite) to induce bone formation anywhere in the body." *Id.* Applicants respectfully disagree.

As the specification shows, simply because a structure has a particular coating on it such as apatite does automatically render it osteoinductive. In the example beginning on page 7 of the specification, a porous body according to the invention was produced and immersed in a 5M-sodium hydroxide aqueous solution, then immersed in distilled water, and then was heated. See specification at page 8, lines 13-18. The resulting implant was osteoinductive. In comparative Example 2, the same sodium hydroxide/water/heat treatment was performed on a circular cylinder comprising a fibrous lump of titanium. *Id.* at page 9, lines 10-16. Such a chemically treated fibre mesh cylinder has a porosity of 40 to 60% and a pore size of 50 to 450 μm, is porous and would be coated by apatite in the living body. Notably, however, the resulting implant of comparative Example 2 was not osteoinductive. See also Fujibayashi et al., *Biomaterials*, 25:443-50 (2004) at pages 444 and 445. Thus, the mere presence of a film or the mere presence of pores that in one circumstance may confer osteoinductivity in one situation does not automatically confer osteoinductivity in all situations.

Moreover, whether or not the film taught by Kokubo has the ability to bond with bones does not speak to whether the resulting implant will has osteoinductivity. The ability to bond with bones is also a feature of osteoconductive implants that form bone only within bony structures. In contrast, osteoinductive implants can form bone even in areas that do not normally contain bone. Kokubo discloses neither osteoinductivity in a location where a living bone does not exist nor any porous structure enabling a lump of titanium to be osteoinductive. Contrary to the Office's assumptions, osteoinductivity is not an inherent result of coating pores in apatite.

Indeed, those of ordinary skill in the art observed osteoinduction only on calcium phosphate ceramics and glass-ceramics until the present invention was made, as shown in the introduction of Habibovic et al., *J. Biomed. Mat. Res.*, 77(4):747-62 (2006), which explains that there are a few reports showing osteoinduction by alumina ceramic and titanium in dogs as well as glass-ceramics. *See* paragraph spanning page 747 and 748. The two reports regarding titanium, references 34 and 35, correspond to the present invention. The report regarding alumina ceramic is doubtful because it contains just an abstract and not a full paper. Until the inventors discovered the present osteoinductive artificial bone, those in the art recognized osteoinduction as a character of calcium phosphate. See Fujibayashi et al., *Biomaterials*, 25:443-50 (2004) at page 446, right column. The inventors were first to find and disclose that a porous body comprised of chemically treated titanium is also osteoinductive.

In sum, the combination of Kawatani with Kokubo does not teach the "osteoinductive artificial bone that is a porous body . . . the body having a pore interconnected in a three-dimensional network . . . the pore penetrating from one end of the body to the other end" recited in independent claim 1. Applicants therefore request that this rejection of claims 1-3, 7-9 and 11 be withdrawn.

The Office rejects claims 4-6 under 35 U.S.C. § 103(a) as allegedly obvious over Kawatani in view of Kokubo and in further view of Johnson et al. (U.S. Patent Application 2001/0053937; "Johnson"). Office Action, page 3. According to the Office, Kawatani teaches a plasma sprayed body/rough film as discussed above. *Id.* The body is allegedly formed by plasma spraying small, irregular titanium particles to a base material or plate. *Id.* at pages 3 and 4. Citing to paragraph 13 of Johnson and to MPEP

§§ 2131.03 and 2144.05, the Office also contends that Kawatani's titanium powder comprises a fine powder having a particle diameter of 20-30 μm and a coarse titanium powder having a particle diameter of 100-300 μm. *Id.* Relying on Johnson for the alleged teaching of cutting porous metal materials to shape them for implantation, the Office concludes that it would have been obvious "modify the plasma sprayed body taught by Kawatani . . . by the shaping/machining/cutting process taught by Johnson . . . in order to be sure that the implant would fit the orthopedic region in question." *Id.* at 4.

Applicant traverse.

As discussed above, the combination of Kawatani and Kokubo does not teach the method of independent claim 3. Specifically, the combination of references does not teach an "osteoinductive artificial bone that is a porous body . . . the body having a pore interconnected in a three-dimensional network . . . the pore penetrating from one end of the body to the other end" and thus cannot teach a method of making such an osteoinductive artificial bone.

Adding Johnson does not compensate for the lack of teaching in Kawatani and Kokubo. The Office refers to paragraph [0048] for the alleged teaching of cutting or machining porous titanium. This paragraph, however, make no mention of cutting porous metal structures let alone structures made of very hard metals like titanium. Instead, this paragraph appears to discuss shaping organic material. Even if the skilled artisan combined Kawatani with Johnson, the resulting structure would be a rough film sitting on the surface of a base material in which the rough film and base material as one structure have been shaped. This does not change the fact that any "pores" that may be present in Kawatani's rough film clearly do not extend through the base material

to the other side. Finally, the Office cites to paragraph [0013] for the alleged teaching of powder particles with the sizes recited in claim 6. This paragraph in Johnson, however, provides no teaching on titanium powder let alone powder with particles of a certain size or of specific diameter ranges such as 20 to 30 μm or 100 to 300 μm.

For these reasons, the combination of Kawatani, Kokubo, and Johnson does not render claims 4-6 obvious. Applicants request that this rejection be withdrawn.

Claim 10 remains rejected under 35 U.S.C. § 103(a) as allegedly obvious over Kawatani in view of U.S. Patent 6,689,170 ("Larsson"). Office Action, page 4. The Office applies Kawatani as discussed above and notes that Kawatani does not teach anodizing the porous body in a electrolytic solution. *Id.* at pages 4 and 5. The Office turns to Larsson for the alleged teaching of an implant for permanent anchorage in bone tissue which is made of titanium with a titanium oxide surface that has been modified by anodization. *Id.* at page 5. The Office concludes that it would have been obvious to combine the method of anodizing in Larsson with the "porous body" taught in Kawatani because anodization increases the oxide thickness on the titanium surface and titanium oxide is suspected to increase the biocompatibility of titanium. *Id.* Applicants traverse.

As discussed above, the combination of Kawatani and Kokubo does not teach the method of independent claim 3. Likewise, the combination of these references cannot teach the method of claim 10. Specifically, the combination of references does not teach an "osteoinductive artificial bone that is a porous body . . . the body having a pore interconnected in a three-dimensional network . . . the pore penetrating from one end of the body to the other end" and thus cannot teach a method of making such an osteoinductive artificial bone.

Adding Larsson does not compensate for the lack of teaching in Kawatani and Kokubo. Kawatani does not teach the osteoinductive artificial bone as described in claim 1 and thus cannot teach a method for producing such an artificial bone as described in claim 10. Like Kawatani, Larsson focuses on making modifications to the surface of a base material. In the case of Larsson, the base material is a titanium disc made from a titanium rod. See col. 11, lines 49-53. As Applicants previously explained, Larsson's anodization of the titanium implant did not give rise to "a pore interconnected in a three-dimensional network with a diameter of 100 to 3000 μm and a hole with a diameter of 50 μm or less on an inner surface of the pore." See Amendment filed November 29, 2007, at pages 14 and 15. Even if it did, the pore could not penetrate "from one end of the body to the other end" as recited in claim 10 because the "pores" would only be formed on the surface of the titanium disc. Thus, because the combination of Kawatani and Larsson do not teach "a method of manufacturing the osteoinductive artificial bone of claim 1" as recited in claim 10, these references do not render claim 10 obvious. Accordingly, Applicants request that the Office withdraw this rejection.

Conclusions

Applicants respectfully requests that this Amendment under 37 C.F.R. § 1.116 be entered by the Office, placing claims 1-11 in condition for allowance. Applicants submit that the proposed amendments of claims 1, 3, and 10 do not raise new issues or necessitate the undertaking of any additional search of the art by the Office. Therefore, this Amendment should allow for immediate action by the Office.

Furthermore, Applicants respectfully point out that the final action by the Examiner presented some new art against Applicants' invention. It is respectfully submitted that the entering of the Amendment would allow the Applicant to reply to the final rejections and place the application in condition for allowance.

Finally, Applicants believe that the entry of the amendment would place the application in better form for appeal, should the Office dispute the patentability of the pending claims.

In view of the foregoing remarks, the claimed invention is not rendered obvious in view of the prior art references cited against this application. Applicants therefore request the entry of this Amendment, the Office's reconsideration and reexamination of the application, and the timely allowance of claims 1-11.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
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Dated: June 25, 2008

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Osteoinduction of porous bioactive titanium metal

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Abstract

This is the first report of bone induction in a non-osseous site by titanium metal, which has long been recognized as a non-bioactive material. After undergoing specific chemical and thermal treatments, porous bioactive titanium induced bone formation without the need of additional osteogenic cells or osteoinductive agents. Four types of titanium implants were implanted in the dorsal muscles of mature beagle dogs, and were examined histologically after periods of 3 and 12 months. Chemically and thermally treated titanium, as well as pure titanium, was implanted either as porous blocks or as fibre mesh cylinders. Bone formation was found only in the chemically and thermally treated porous block implants removed after 12 months. The present study shows that even a non-soluble metal that contains no calcium or phosphorus can be an osteoinductive material when treated to form an appropriate macrostructure and microstructure. This finding may elucidate the nature of osteoinduction, and lead to the advent of epochal osteoinductive biomaterials for tissue regeneration.

Keywords: Osteoinduction; Titanium; Porous; Metal surface treatment

1. Introduction

Bioactive materials, including hydroxyapatite, Bioglass, and Glass-ceramic AW, have osteoconductive abilities, and can directly bond to living bone via an apatite layer [1–3].

It is recognized that biomaterials do not have an osteoinductive character in the absence of additional osteoinductive agents, such as bone morphogenetic proteins (BMPs). However, certain calcium phosphate biomaterials have recently been reported to be osteoinductive if they possess a specific porous structure [4–8]. These calcium phosphate biomaterials induce bone formation at extra-skeletal sites without the need for additional osteogenic cells or BMP. Implantation in soft tissue provides conclusive proof of bone induction by a biomaterial. However, the mechanism of osteoinduction by calcium phosphate ceramics is not clear. As osteoinductive biomaterials contain calcium and phosphorus, the important factors for osteoinduction are thought to be (i) the chemical composition of the biomaterial, (ii) the specific dissolution properties of the biomaterial, and (iii) the surface morphology of the biomaterial. From a clinical point of view, the ideal biomaterial acting as a bone substitute should possess osteoconductive and osteoinductive ability, and it should have superior mechanical properties. Titanium metal is considered a bioinert material, and is used for scaffolds when loaded with BMP to induce ectopic bone formation [9–11]. In a previous study, we showed that titanium metal could be converted into an osteoconductive material through specific chemical and thermal treatments [12]. This bioactive titanium shows a superior in vitro apatite-forming ability, and is able to directly bond to living bone in vivo [13,14]. Although the bioactive titanium has an osteoconductive ability, it does not possess any osteoinductive ability.

Recently, we have developed an interconnective porous titanium block using a plasma-spray technique, and this porous titanium has been successfully subjected

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to chemical and thermal treatments [15]. In this paper, we describe our development from titanium metal of an osteoinductive material that contains no calcium and phosphorus, and discuss the mechanism of material-induced osteogenesis, along with the relationship between osteoconduction and osteoinduction.

2. Material and methods

2.1. Implants

Four types of titanium implants were prepared. Bioactive titanium was prepared by a chemical treatment (immersion in 5 M aqueous NaOH solution at 60°C for 24 h), followed by a hot water treatment (immersion in distilled water at 40°C for 48 h), followed by a thermal treatment (heating to 600°C at a rate of 5°C/min, maintained at 600°C for 1 h, and then allowed to cool at the natural rate of the furnace). Pure titanium was used as a control [16,17]. These materials were implanted as porous blocks ($5 \times 5 \times 7\,mm^3$, porosity = 40–60%, pore size = 300–500 μm). The titanium was supplied by Kobe Steel Ltd., Kobe, Japan (see Fig. 1A). The titanium fibre mesh cylinders (diameter = 4 mm, length = 11 mm, porosity = 40–60%, pore size = 50–450 μm) were supplied by the Kyocera Corporation, Kyoto, Japan (see Fig. 1B). The porous titanium blocks were manufactured as follows. A macro-porous titanium layer was formed on the titanium substrate by plasma spraying commercial pure titanium powder with a particle size of 50–200 μm. Blocks were cut from the porous layer using an electric discharge. The tensile strength and the bending strength of the porous titanium were 80.0 and 91.9 MPa, respectively. The details of the manufacturing process and mechanical properties for porous titanium will be reported elsewhere. The titanium fibre mesh implants were manufactured by compacting a single 250-μm fibre into a die to a porosity of 50%, followed by vacuum sintering.

2.2. Animal experiments

Four types of sample were implanted in the dorsal muscles of mature beagle dogs (weight = 10–11 kg), for periods of 3 and 12 months. Six beagle dogs were used in this study. The animals were anaesthetized by intramuscular administration of ketamine hydrochloride (50 mg/kg), followed by diazepam (5 mg) and atropine sulphate (0.5 mg) without endotracheal intubation. Just before the operation, a dose of 10 mg/kg pentobarbital sodium was injected intravenously. During the operation, the dogs received an intravenous infusion of saline containing isepamicin sulphate for antibiotic. The operations were performed under the usual sterile conditions. After the skin and fascia had been incised, muscle pouches were carefully made at the dorsal muscle to limit any bleeding. Each sample was implanted in each pouch separately to prevent inter-sample contact. Twenty-four samples were implanted. The Kyoto University guidelines for animal experiments were observed in this study.

2.3. Assessment of the morphology of the porous titanium

Before implantation, the surface morphology of the samples was examined using scanning electron microscopy (SEM) (S-4700, Hitachi Ltd., Tokyo, Japan). The interconnective structure of the porous titanium was examined using a micro-CT analyser. A high-resolution X-ray CT scanner (Andrex MX-4), using a micro-focus X-ray tube (Hamamatsu Photonics C8033) with a high-resolution computing system was used for three-dimensional (3D) reconstruction and analysis. The micro-focus X-ray tube had a focal spot of about 5 μm. The 3D structures of the materials were imaged and reconstructed from hundreds of 2D sectional CT images,

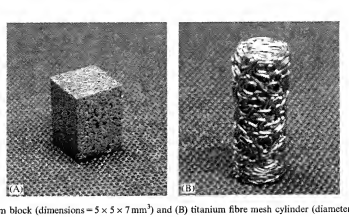

Fig. 1. (A) Porous titanium block (dimensions = $5 \times 5 \times 7\,mm^3$) and (B) titanium fibre mesh cylinder (diameter = 4 mm, length = 11 mm).

which were obtained in a single scan over a 360° rotation about the sample. The 2D image detector used was an image intensifier equipped with a digital CCD camera.

2.4. Assessment of the in vitro bioactive ability of the porous titanium

The bioactive ability of the samples was examined by soaking them in a simulated body fluid (SBF) having a pH = 7.40, with the following ion concentrations: $Na^+ = 142.0$, $K^+ = 5.0$, $Ca^{2+} = 2.5$, $Mg^{2+} = 1.5$, $Cl^- = 147.8$, $HCO_3^- = 4.2$, $HPO_4^{2-} = 1.0$, and $SO_4^{2-} = 0.5$ mM [18]. The samples were soaked in 30 ml of the SBF for 7 days at 36.5°C, then removed from the SBF, washed with acetone, and dried on a clean bench. After being soaked in the SBF, the surface of the samples was examined by SEM, and any apatite formed was identified from its morphology. The in vivo bioactivity (osteoconductive ability) of these ceramics is precisely mirrored by their in vitro apatite-forming ability in an SBF [19].

2.5. Preparation of the samples and histological examination

After removal, the samples were fixed using a 10% phosphate-buffered formalin solution at pH = 7.40 for 7 days, and then dehydrated in serial concentrations of ethanol of 70%, 80%, 90%, 99%, 100% and 100% v/v every 3 days. Then, the samples were embedded in polyester resin. A band saw (BS-3000CP, Exact cutting system, Norderstedt, Germany) was used to cut 250-μm thick sections, which were then polished using diamond paper, and coated with a thin layer of carbon for observation using a backscattered SEM attached to an EDX micro-analyser.

Some sections taken from the samples were ground to a thickness of 20 μm using a diamond lap disk (MG-4000, Exact grinding system, Norderstedt, Germany) and hand brushing, and then surface-stained with toluidine blue and Giemsa for examination using an optical microscope.

3. Results

3.1. Surface morphology before implantation

SEM examination of the samples before implantation revealed that two types of porous macrostructure were present in the block and cylinder implants (see Figs. 2A and B). The porous macrostructure of the block-type implant was more complex than that of the cylinder-type implant. High magnification revealed that micro-porous structures were recognizable in the macro-pores of both the chemically and thermally treated titanium implants (Figs. 2C and D). On the other hand, the micro-surface of both of the non-treated titanium implants was smooth (Figs. 2E and F). Micro-CT and 3D reconstruction image analysis clearly demonstrated the 3D interconnectivity of the porous structures (Figs. 3A and B). From the micro-CT images, the average porosity of the porous titanium samples was calculated to be 38.8%.

3.2. Assessment of the in vitro bioactive ability

After the titanium blocks had been soaked in the SBF, apatite deposits could be recognized on all the chemically and thermally treated titanium blocks and cylinders within a 7-day period. On the other hand, no surface morphological changes were observed in the non-treated titanium implants after soaking in the SBF for a 7-day period. These results indicate that the chemically and thermally treated titanium implants possessed an in vitro apatite-forming ability and an in vivo osteoconductive ability.

3.3. Histological examination

Both SEM and optical microscopy showed that no bone formation had occurred in all the samples removed1 after 3 months. After 12 months, however, bone formation was found in the chemically and thermally treated porous block implants. Bone formation was observed in all three porous bioactive titanium samples after 12 months (Table 1). Optical microscopy showed mineralized, newly formed bone that stained well, and had a lamellar structure at the surface of the inner pores (see Fig. 4A). However, no bone formation could be recognized at the outer surface of the porous blocks. Normal bony structures with osteocytes embedded in the lacunae and canalicular network were observed in the Giemsa stained samples (see Fig. 4B). SEM and EDX analysis showed that new bone had bonded to the titanium surface directly, and that it contained calcium and phosphorus (Figs. 5A, B and C). The observed Ca/P ratio was 1.66% w/w. These findings indicate that new bone appeared on the surface of the bioactive titanium within the pores and extended throughout the porous network. These results also demonstrate the interconnectivity of the porous structure, and that the correct size of pore to allow cells and tissues to invade had been attained. No crystal formation or pathological calcifications were observed.

3.4. Quantitative data of new bone in porous titanium

The quantity of newly formed bone was determined using an Image-Pro plus image analyser (Media Cybernetics, USA). Although bone formation was

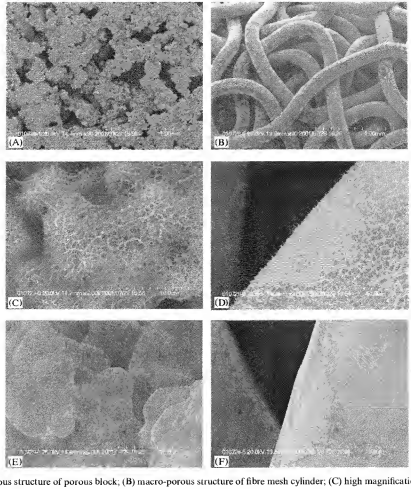

Fig. 2. (A) Macro-porous structure of porous block; (B) macro-porous structure of fibre mesh cylinder; (C) high magnification view of the surface of porous block after chemical and thermal treatments; (D) surface of the fibre mesh cylinder after chemical and thermal treatments; (E) surface of the porous block without any surface treatment; and (F) surface of the fibre mesh cylinder without any surface treatment. The macro-porous structure of the porous blocks was more complex than that of the fibre mesh cylinder. After the chemical and thermal treatments, a 3D micro-porous structure was formed in the macro-pores.

found in all the bioactive porous titanium samples harvested at 12 months, only a small mass of bone had formed at the centre of the implants. In all, $16.2 \pm 7.5\%$ new bone had formed in the pores that covered $23.2 \pm 5.5\%$ of implant area.

4. Discussion

The porous bioactive titanium prepared using specific chemical and thermal treatments induced bone formation at non-osseous sites without the need for additional osteogenic cells or osteoinductive agents.

Ripamonti [20] demonstrated the induction of bone in coral-derived porous hydroxyapatite, when it was implanted intramuscularly in baboons. He concluded that the hydroxyapatite substratum may function as a solid-phase domain for the anchorage of BMPs. Yuan et al. [21] reported the osteoinduction of two types of calcium phosphate ceramics that were similar in their chemical and crystallographic structures, but had different microstructures. They concluded that the micro-pores on the macro-pore walls of these materials were important for osteoinduction of calcium phosphate ceramics. In addition, they reported on bone induction by a porous glass-ceramic [22]. Bone induction by glass-ceramics, together with bone induction by other calcium phosphate-based biomaterials, indicates that osteoinduction may be a common phenomenon or property exhibited by calcium phosphate biomaterials.

From the results of our study, we can first hypothesize that the complex macro-porous structure plays an important role in material-induced osteogenesis. Although the chemically and thermally treated cylinders

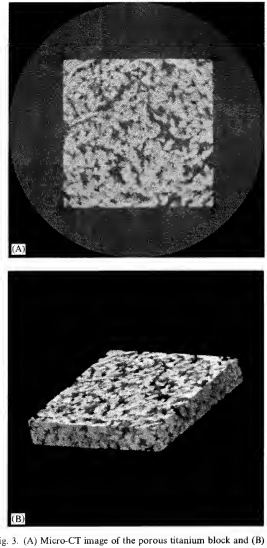

Fig. 3. (A) Micro-CT image of the porous titanium block and (B) 3D picture of a reconstructed CT. The porous structure is interconnected in three dimensions.

Table 1
Bone formation by four types of titanium implant in dog muscle

	3 months	12 months
TP	0/3	0/3
TF	0/3	0/3
BTP	0/3	3/3 (100%)
BTF	0/3	0/3

TP, non-treated porous titanium; TF, non-treated fibre mesh, BTP, treated porous titanium; BTF, treated titanium fibre mesh.

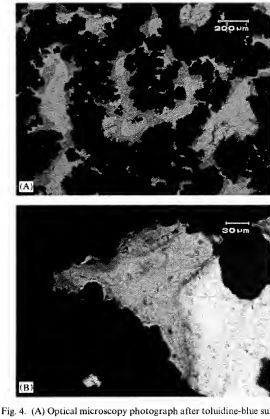

Fig. 4. (A) Optical microscopy photograph after toluidine-blue surface staining (original magnification = × 40). Well-stained, massive newly formed bone with a lamella structure was observed on the inner surface of the pores. (B) Normal bony structure with osteocytes embedded in the lacunae and canalicular network (Giemsa surface staining, magnification = × 200).

showed apatite formation in vitro, they did not show osteoinduction in vivo. The interconnective macroporous structure of the porous titanium block, which was more complex than that of the fibre mesh cylinders, was effective for osteogenesis. Osteogenic cells and agents may be successfully trapped in such macroporous structures. In the present study, new bone formation was observed only in the inner pores of the blocks, and not in the outer pores. This observation indicates that the threshold for specific growth factors or ionic concentrations could be reached easily, and so inductive osteogenesis could occur in the inner pores. We suggest the importance of the ionic concentration for inducing an osteoinductive ability, because the evidence provided by porous calcium phosphate ceramics shows that they induce osteogenesis earlier than titanium implants which contain no calcium and phosphorus. In our in vitro study, the apatite-forming ability of the chemically and thermally treated titanium was the same as that of the calcium phosphate ceramics. That is, the in vivo osteoconductive ability of bioactive titanium is believed to be the same as that of hydroxyapatite or bioactive glasses. However, indeed the in vivo osteoinductive ability of porous bioactive

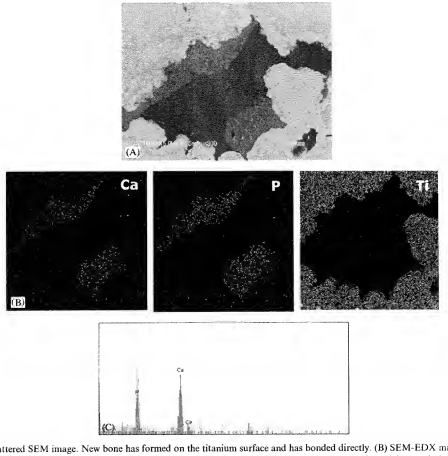

Fig. 5. (A) Backscattered SEM image. New bone has formed on the titanium surface and has bonded directly. (B) SEM-EDX mapping images (Ca, calcium; P, phosphorous; Ti, titanium). (C) EDX analysis of newly formed bone. The bony structure contains calcium and phosphorous

titanium was not the same as those of porous calcium phosphate ceramics. The ideal ionic concentrations required to induce osteoinduction in the inner pores have yet to be determined. We have carried out an osteoinductive study using biomimetic apatite-coated porous bioactive titanium that contained calcium and phosphorus. Those results (which we have not reported here) strongly suggested the importance of Ca and P ions in the inducement of osteogenesis.

A second point to note is that, although the non-treated blocks also possessed a similar macro-porous interconnective structure, they did not exhibit an osteoinductive nature. The surface 3D micro-porous structure, which was formed by the chemical and thermal treatments, plays an important role in osteogenesis. From our previous study, the 3D micro-porous structure was related to the in vitro apatite formation of chemically and thermally treated titanium. An in vitro apatite-forming ability may be a necessary prerequisite for biomaterials to be osteoinductive materials in vivo. In the current study, apatite may have been spontaneously deposited on the surface of the bioactive titanium. That is, bioactive titanium spontaneously formed a very thin coating of calcium phosphate ceramic in vivo. As BMPs demonstrate a high affinity for calcium phosphates [23,24], this calcium phosphate self-coating may have a stimulatory effect on ectopic bone formation by BMPs.

The interconnective porous structure may play an important role in osteogenesis. In this work, the interconnection of the porous structure of the porous titanium block fabricated using a plasma-spray technique was established from the histological examinations. From the observed bone formation in the inner pore regions, the distribution of the inter-pore connections was adequate to enable cells to invade. The optimal pore diameter for in vivo osteoconduction is thought to be in the range 150–500 μm [25,26]. Moreover, the interconnectivity of a porous structure has been shown to be effective for the osteoconduction of sintered porous hydroxyapatite, by allowing for the invasion of cells and tissues deep into the pores [27].

Although many hypotheses could be postulated from the results of this study and the osteoinduction of other calcium phosphate ceramics, the true mechanism of osteoinduction by porous bioactive titanium is not understood. To elucidate such problems, we must carry out more studies, including other bone-specific stains and immuno-stains. In this study, we examined the new bone using Giemsa and toluidine-blue surface staining and SEM. Because decalcification of porous titanium was not possible, the bone staining techniques used to characterize the new bone may have had limitations. To alleviate this, we prepared thin, 20 μm thick, undecalcified sections. The preparation of these thin undecalcified sections containing titanium was difficult. Histomorphometric analysis demonstrated a discrepancy between the porosity results obtained using the micro-CT image (38.3%) with those obtained using an optical microscopic image (23.2%). This result may be caused by the thickness of the samples for microscopic image.

This paper also represents the first report of interconnective structured titanium metal. In general, the fabrication of porous metals is difficult, and this is especially so for titanium. The porous structure is useful for tissue regeneration in relation to its use as a scaffold for growth factors or osteogenic cells. However, the mechanical properties of porous ceramics are too poor for clinical use under load-bearing conditions. Porous titanium has a high enough mechanical strength for use under load-bearing conditions. We also believe that a combination of bioactive porous titanium and growth factors or osteogenic cells is useful for bone tissue engineering under load-bearing conditions.

5. Conclusions

The present study has shown that even a metal that possesses no dissolution properties and contains no calcium and phosphorus can become an osteoinductive material when made to possess a specific macrostructure and microstructure. That is, all osteoconductive materials that induce an apatite layer in the body may have the potential to be osteoinductive materials when they possess a specific porous structure. This observation may help elucidate the nature of osteoinduction, and lead to the advent of epochal osteoinductive biomaterials for tissue regeneration.

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References

[1] Kokubo T. Bioactive glass ceramics: properties and applications. Biomaterials 1991;12:155–63.
[2] Hench LL. Bioactive materials: the potential for tissue regeneration. J Biomed Mater Res 1998;41:511–8.
[3] Neo M, et al. A comparative study of ultrastructures of the interfaces between four kinds of surface-active ceramic and bone. J Biomed Mater Res 1992;26:1419–32.
[4] Ripamonti U. Osteoinduction in porous hydroxyapatite implanted in heterotopic sites of different animal models. Biomaterials 1995;17:31–5.
[5] Yang Z, et al. Osteogenesis in extraskeletally implanted porous calcium phosphate ceramics: variability among different kinds of animals. Biomaterials 1996;17:2131–7.
[6] Yuan H, Li Y, de Bruijn JD, de Groot K, Zhang X. Tissue responses of calcium phosphate cement: a study in dogs. Biomaterials 2000;21:1283–90.
[7] Yuan H, et al. Bone formation induced by calcium phosphate ceramics in soft tissue of dogs: a comparative study between porous α-TCP and β-TCP. J Mater Sci 2001;12:7–13.
[8] Zhang X. The osteoinductivity of Ca-P biomaterials and the potential in clinic. Proceedings of the Asian BioCeramics, Gyeongju, Korea, 2002. p. 21–4.
[9] Cole BJ, et al. Use of bone morphogenetic protein 2 on ectopic porous coated implants in the rat. Clin Orthop 1997;345: 219–28.
[10] Vohof JWM, Spauwen PHM, Jansen JA. Bone formation in calcium-phosphate-coated titanium mesh. Biomaterials 2000; 21:2003–9.
[11] Ferretti C, Ripamonti U. Human segmental mandibular defects treated with naturally derived bone morphogenetic proteins. J Craniofac Surg 2002;13:434–44.
[12] Kokubo T, Miyaji F, Kim HM, Nakamura T. Spontaneous apatite formation on chemically surface treated Ti. J Am Ceram Soc 1996;79:1127–9.
[13] Nishiguchi S, et al. The effect of heat treatment on bone-bonding ability of alkali-treated titanium. Biomaterials 1999;20:491–500.
[14] Fujibayashi S, et al. Bioactive titanium: effect of sodium removal on the bone-bonding ability of bioactive titanium prepared by alkali and heat treatment. J Biomed Mater Res 2001;56:562–70.
[15] Kim HM, Kokubo T, Fujibayashi S, Nishiguchi S, Nakamura T. Bioactive macroporous titanium surface layer on titanium substrate. J Biomed Mater Res 2000;52:553–7.
[16] Uchida M, Kim HM, Kokubo T, Fujibayashi S, Nakamura T. Structural dependence of apatite formation on titania gel in a simulated body fluid. J Biomed Mater Res 2003;64:164–70.
[17] Uchida M, Kim HM, Kokubo T, Fujibayashi S, Nakamura T. Effect of water treatment on the apatite-forming ability of NaOH-treated titanium metal. J Biomed Mater Res (Appl Biomater) 2002;63:522–30.
[18] Kokubo T, Kushitani H, Sakka S. Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A-W. J Biomed Mater Res 1990;24:721–34.
[19] Fujibayashi S, Neo M, Kim HM, Kokubo T, Nakamura T. A comparative study between in vivo bone ingrowth and in vitro apatite formation on Na_2O–CaO–SiO_2 glasses. Biomaterials 2003;24:1349–56.
[20] Ripamonti U. The morphogenesis of bone in replicas of porous hydroxyapatite obtained from conversion of calcium carbonate exoskeletons of coral. J Bone Jt Surg 1991;73(A):692–703.
[21] Yuan H, et al. A preliminary study on osteoinduction of two kinds of calcium phosphate ceramics. Biomaterials 1999;20: 1799–806.
[22] Yuan H, de Bruijn JD, Zhang X, van Blitterswijk CA, de Groot K. Bone induction by porous glass ceramic made from

Bioglass® (45S5). J Biomed Mater Res (Appl Biomater) 2001; 58:270–6.

[23] Uludag H, D'Augusta D, Palmer R, Timony G, Wozney J. Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model. J Biomed Mater Res 1999;46:193–202.

[24] Vehof JWM, et al. Ectopic bone formation in titanium mesh loaded with bone morphogenetic protein and coated with calcium phosphate. Plast Reconstr Surg 2001;108:434–43.

[25] Hulbert SF, Morrison SJ, Klawitter JJ. Tissue reaction to three ceramics of porous and non-porous structures. J Biomed Mater Res 1972;6:347–74.

[26] Flatley TJ, Lynch KL, Benson M. Tissue response to implants of calcium phosphate ceramic in the rabbit spine. Clin Orthop 1983;179:246–52.

[27] Tamai N, et al. Novel hydroxyapatite ceramics with an interconnective porous structure exhibit superior osteoconduction in vivo. J Biomed Mater Res 2002;59:110–7.

Osteoinduction by biomaterials—Physicochemical and structural influences

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Abstract: Osteoinduction by biomaterials has been shown to be a real phenomenon by many investigators in the last decade. The exact mechanism of this phenomenon is, however, still largely unknown. This *in vivo* study in goats was performed to get insight into processes governing the phenomenon of osteoinduction by biomaterials and had four main goals: (i) to further investigate the influence of physicochemical properties and structure on biomaterial osteoinductive potential, (ii) to investigate the influence of implant size on the amount of induced bone, (iii) to investigate implantation site dependence, and (iv) to investigate changes occurring on the surface of the material after implantation. Intramuscular implantations of four different biphasic calcium phosphate ceramics, consisting of hydroxyapatite and β-tricalcium phosphate and a carbonated apatite ceramic, indicated that, for a maximal osteoinductive potential, there is an optimal specific surface area for each material type. It was further shown that a decrease of the implant size with a half significantly decreased the relative amount of induced bone. In addition, subcutaneous implantation did not give rise to ectopic bone formation in any of the animals, while bone was induced in most animals intramuscularly. Analysis of the surfaces of the materials after subcutaneous implantation inside diffusion chambers indicated that the increased specific surface area leads to more surface reactivity, which is hypothesized to be essential for osteoinductivity by biomaterials. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 747–762, 2006

Key words: osteoinduction; biomaterials; mechanism; biphasic calcium phosphate ceramic; carbonated apatite ceramic

INTRODUCTION

First reports on bone and cartilage formation after implantation of devitalized tissues[1–3] and tissue extracts[4] ectopically (i.e., in the absence of bone cells) have already been published in the forties and fifties of the previous century. In the late sixties, Urist defined osteoinduction as "the mechanism of cellular differentiation towards bone of one tissue due to physicochemical effect or contact with another tissue."[5] A year later, Friedenstein defined osteoinduction as "the induction of undifferentiated inducible osteoprogenitor cells that are not yet committed to the osteogenic lineage to form osteoprogenitor cells."[6] Later work of Urist and Reddi[7–10] resulted in finding bone morphogenetic proteins (BMPs), which were proposed to irreversibly induce differentiation of perivascular mesenchymal-type cells into osteoprogenitor cells to finally form cartilage and bone.[10] Although osteoinduction by certain types of devitalized tissues and thus BMPs has been extensively investigated, the exact mechanism is not fully understood yet.

The search for the explanation of the mechanism of osteoinduction was further complicated by Winter's and Simpson's early finding of ectopic bone formation after implantation of a polyhydroxylethylmethacrylate sponge that neither contained nor produced BMPs.[11] In this study, authors observed that osteoinduction was preceded by the *in vivo* calcification of the polymeric sponge. Up to now, many groups have reported osteoinduction by various synthetic biomaterials such as synthetic hydroxyapatite (HA) ceramic in dogs,[12–16] coral-derived HA ceramic in dogs, monkeys, and baboons,[13,17,18] α-tricalcium phosphate (α-TCP), β-TCP, biphasic calcium phosphate (BCP), α-pyrophosphate, and β-pyrophosphate ceramics.[18–28] Besides CaP bulk ceramics, ectopic bone formation was also found in CaP cements[19,29] and in various porous implants coated with biomimetic octacalcium phosphate.[21,30,31] In addition to the CaP-containing

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biomaterials, there are a few reports showing osteoinduction by glass ceramic,[32] alumina ceramic,[33] and titanium in dogs,[34,35] all of which have the ability to calcify in a CaP-rich environment. These reports all give further evidence that osteoinduction by biomaterials is a real phenomenon.

Although both BMPs and biomaterials can lead to ectopic bone formation, it is unknown whether they (partially) follow the same pathway. In contrast to the mainly endochondral, BMP-triggered osteoinduction,[8] cartilage formation as a precursor of the final bone formation has never been observed in osteoinduction by biomaterials without added BMPs,[17,19,20,26,36] and is thus intramembraneous. Another difference between BMP- and biomaterials-guided osteoinduction is their incidence in different animal models: bone is abundantly induced in soft tissue of rats and mice by using BMP-2 and BMP-7 (OP-1)[37–39] but hardly by synthetic biomaterials.[12,40–43] Ripamonti and coworkers suggested the influence of BMPs in osteoinduction by biomaterials, but did not prove this hypothesis by experimental data.[36,44]

During the last decade, experiments by various investigators in large animal models have shown parameters that are of great importance in the process of osteoinduction by biomaterials. For instance, it has been shown that the presence of concavities or macropores is one of the prerequisites for osteoinduction.[19,36] Furthermore, the presence of micropores, by which the specific surface area of the material is increased, seems to be essential as well.[19,20,25–27,33,34] It has been proposed that the increased specific surface area leads to more surface reactivity in terms of dissolution and reprecipitation of a biological apatite layer, which possibly coprecipitates relevant endogenous proteins, which in turn initiate the differentiation of multipotent cells into the osteogenic lineage.[20]

The present *in vivo* study was performed so as to get more insight into the processes governing the phenomenon of osteoinduction by biomaterials, by attempting to achieve following goals:

i. to further investigate the influence of physicochemical properties and structure on biomaterial osteoinductive potential. We compared five CaP ceramics, four of which were biphasic, consisting of HA and β-TCP, with slightly varying chemical composition and large variations in microstructure, and a carbonated apatite (CA) ceramic, that is, based on its chemistry, closer to bone mineral than the BCP ceramics;
ii. to investigate the influence of implant size on the amount of induced bone. The availability of the implant for cells and nutrients infiltration, as well as possible micromotion inside the implant could be influenced by its size;
iii. to investigate the influence of implantation site on osteoinduction by biomaterials. Implantation site dependence, that is, comparison of intramuscular and subcutaneous implantation, could shed light upon the importance of vascularization in the process of osteoinduction; and
iv. to investigate changes occurring on the surface of an osteoinductive and a nonosteoinductive ceramic after implantation inside diffusion chambers. The idea behind this part of the study was to test the earlier given hypothesis that the high specific surface area of osteoinductive biomaterials, which causes a high surface reactivity, is essential for osteoinduction by biomaterials.[20]

MATERIALS AND METHODS

Implants

In this study, five ceramic types were investigated: biphasic calcium phosphate A (BCPA), BCPB, BCPC, BCPD, and CA. The four BCP ceramics were used to investigate the influence of variations of physicochemical properties, achieved by differences in preparation methods, on the osteoinductive potential of the ceramics. The osteoinductive properties of the rather novel CA bulk ceramic are interesting because, based on its chemical composition, CA ceramic resembles the bone mineral composition more than BCP ceramics do.

BCPA and BCPB implants were prepared by using the hydrogen peroxide foaming method as published earlier.[26] For the preparation of the ceramic, in-house made BCP powder was used. Porous green bodies were produced by mixing this powder with 2% hydrogen peroxide solution (1.0 g powder/1.2 ± 0.05 mL solution) and naphthalene particles (710–1400 μm; 100 g powder/30 g particles) at 60°C. The naphthalene was then evaporated at 80°C and the green porous bodies were dried. They were divided into two groups and sintered at 1150°C (BCPA) and 1300°C (BCPB), respectively, for 8 hours.

BCPC, BCPD, and CA are novel ceramics, developed in the course of the EU "IntelliScaf" project (G5RD-CT-2002–00697).

A lathe was used to produce cylinders with a size of ø 6.5 × 10 mm^3 (all ceramic types) and ø6.5 × 5 mm^3 (BCPA and BCPB). All implants were cleaned in ultrasonic baths and sterilized by gamma irradiation.

Composition and crystal structure of the ceramics were determined by using fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD). HA/β-TCP weight ratios in the BCP ceramics were determined by comparing the BCP XRD patterns with the calibration patterns prepared from the powders with the known HA/β-TCP weight ratios.

Prior to implantation, ultrastructure of all ceramics was characterized by an environmental scanning electron microscope (ESEM) in the secondary electron mode. ESEM, coupled with an image analysis system, was used to determine macroporosities (pore diameter > 10 μm) of the ceramics. In

TABLE I
Overview of the Total Amounts of the Implanted Ceramics

Implant	IM (12 weeks)	SC (12 weeks)	SC (4 weeks)	SC (2 weeks)	SC (1 week)
BCPA	10				
BCPB	10				
BCPC	10				
BCPD	10				
CA	10				
BCPAh	10	10			
BCPBh	10	10			
DC BCPA			20	20	20
DC BCPB			20	20	20

DC, diffusion chamber.

addition, total porosities, macroporosities, and microporosities (pore diameter < 10 μm) as well as average pore sizes and pore size distributions were determined by using a mercury intrusion porosimeter. Specific surface areas of the ceramics were determined from the mercury intrusion porosimeter results, as the cumulative surface area (m^2/g) when all pores were filled with mercury.

After implantation, macroporosities of the ceramics were measured again by using histological sections.[20,21] First, the labels of the sections were covered. Then, high-resolution (300 dpi), low-magnification digital micrographs were made of these sections. Image analyses on the pseudocolored micrographs were carried out with a computer-based image analysis system. Prior to measurements, the system was geometrically calibrated with an image of a cylinder of known dimensions. A program was developed to quantify macroporosities of the ceramics. The macroporosity was determined as: [(total implant surface−scaffold surface)/total implant surface] × 100%.

Animals and implantation

This study was approved by the Dutch Animal Care and Use Committee. In total, 10 adult Dutch milk goats (18–30 months) were used. The animals were housed in the Central Animal Laboratory Institute, Utrecht, The Netherlands, at least 4 weeks prior to surgery.

Surgical procedures were performed under general inhalation anesthesia of the animals preceded by an intravenous injection of Thiopental (Nesdonal, ±400 mg/70 kg of body weight, on indication).

For the comparison of osteoinductive potentials of the five used ceramic types, all materials were implanted intramuscularly in all animals. For intramuscular implantation, fascia incisions were created in the paraspinal muscles (L1–L3). Using blunt dissection, intramuscular pockets were created, and filled with one of the above ceramic cylinders (ø 6.5 × 10 mm^3): BCPA, BCPB, BCPC, BCPD, and CA. Subsequently, fascias were closed with nonresorbable sutures to facilitate implant localization at explantation. The skin was closed in two layers.

For investigation of implantation site and implant size dependence on the amount of induced bone, we used BCPA and BCPB ceramics as a model. For the investigation of the implant size dependence, in addition to the above described intramuscularly implanted cylinders with the size ø 6.5 × 10 mm^3, smaller cylinders (ø 6.5 × 5 mm^3) of both BCPA and BCPB were implanted.

To investigate the implantation site dependence, BCPA and BCPB cylinders were, besides intramuscularly, also implanted subcutaneously. For subcutaneous implantations, separate blunt incisions were made in the skin of the back area, one cylinder with a size of ø 6.5 × 5 mm^3 of each BCPA and BCPB ceramic was inserted, after which the skin was closed in two layers.

Pain relief was given by Durogesic 25 (fentanyl transdermal system CII patches).

In all animals, 8, 10, and 11 weeks after first surgery, cylinders (ø 6.5 × 10 mm^3) of BCPA and BCPB ceramics inside diffusion chambers were implanted subcutaneously by following the same procedure as described above. Because of a relatively small impact on animals, local anesthesia (subcutaneously injected lidocaine) was used during these surgeries. Diffusion chambers in which the ceramics were placed prior to implantations consisted of a Plexiglas ring on both ends of which ø 0.45 μm Durapore® HV filters were pasted using the MF cement for diffusion chamber (Diffusion chamber kit, Millipore) as described previously.[45] Implantation of materials inside these semipermeable chambers allows the diffusion of macromolecules and ions into the chamber but blocks the invasion of host cells and tissues.

Table I gives an overview of total amounts of the implanted ceramics.

To visualize the dynamics of bone growth, the goats received sequential fluorochrome labels at 4 weeks (Calceine green, 10 mg/kg, i.v.), 6 weeks (Oxytetracycline, 32 mg/kg, i.m.), and 8 weeks (Xylenol orange, 80 mg/kg, i.v.).[27,46,47]

Twelve weeks after first implantations, each animal was sacrificed by an overdose of pentobarbital (Euthesaat) and potassium chloride.

Retrieval of the implants, histology, histomorphometry, and material analysis

The implants with surrounding tissue were explanted by sharp dissection and fixed in Karnovsky's fixative. They were dehydrated in a graded ethanol series (70–100%) and transferred into a methylmethacrylate solution that poly-

merized at 37°C within 1 week. Longitudinal sections (10–15 μm in thickness) were made by using the modified interlocked diamond saw. Sections were stained with 1% methylene blue and 0.3% basic fuchsine after etching with HCl/ethanol mixture and then qualitatively investigated by using a light microscope (LM). Additional sections were left unstained for epifluorescence microscopy with an LM equipped with a quadruple filter block (XF57, dichroic mirror 400, 485, 558, and 640 nm).

For histomorphometry, the same image analysis method was used as previously described for the measurement of material macroporosities postimplantation. In the computer-based image analysis system, a program was developed to quantify different parameters concerning bone formation:

a. Percentage of bone occupying available pore area (area%b/pore)

b. Percentage of available scaffold outline (in 3D surface of the scaffold) in contact with bone: [%contact = (bone-scaffold contact length/scaffold outline length) × 100%] in the total area of the implant (%b.cont).

The first parameter was chosen to allow for comparison with previous studies. The second parameter was chosen based on fact that when comparing the performances of different materials, it seems more appropriate to relate the new bone formation to available scaffold, rather than to available pore space. Furthermore, contact percentage is more sensitive for early bone apposition, which generally first occurs on the ceramic surface and has relatively low volume.

After explantation, BCPA and BCPB ceramics, which were implanted subcutaneously inside diffusion chambers, were removed from the chambers and dried at 60°C in air for 2 days. They were then powdered in a mortar, and the powder was homogenized. One part of the powder was used to perform FTIR and XRD analyses. Other part was used for thermogravimetry analysis (TGA). TGA analyses were performed on samples weighing 10–20 mg at a heating speed of 10°C/min. Weight loss between 30°C and 200°C, 200°C and 450°C, and 450°C and 900°C were associated to water, organic compounds, and CO_2, respectively. From the weight loss, weight percentages of organic compounds and CO_3 were calculated.

Statistical analysis

Statistical calculations were done with the SPSS 12.0 software.

In agreement with previous studies, intramuscularly implanted ceramics showed large variances between the individual animals.[20,21] Hence, the distribution of the data was not normal and that is why the nonparametric Wilcoxon signed rank test[18] for paired comparisons was used to perform the statistical analyses.

For the quantitative TGA data, the two-sided, paired Student's t test was used to analyze differences between BCPA and BCPB.

For both tests, the significance level was set at $p = 0.05$.

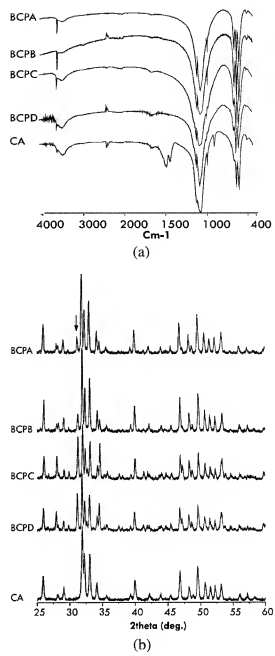


Figure 1. FTIR spectra (a) and XRD patterns (b) of BCPA, BCPB, BCPC, BCPD, and CA ceramic. Arrow indicates main β-TCP peak in BCP ceramics.

RESULTS

Material characterization

FTIR spectra and XRD patterns of the four BCP and the CA ceramic are given in Figure 1(a,b). All BCP ceramics had similar, biphasic structure, consisting of HA and β-TCP. However, their HA/TCP weight ratios differed: BCPA and BCPB ceramics consisted of

80 ± 3 wt % HA and 20 ± 3 wt % β-TCP, while the contents of HA and β-TCP in BCPC and BCPD were 70 ± 5 wt % and 30 ± 5 wt %, respectively, which is also illustrated by the differences in the height of the main β-TCP peak (at 2θ = 31.2°) in the BCP XRD patterns [Fig. 1(b)]. The FTIR spectrum of the CA ceramic exhibits typical CA structure with distinct bands assigned to CO_3^{2-} group at 1458 cm^{-1}, 1416 cm^{-1}, and 872 cm^{-1} [Fig. 1(a)]. CO_3^{2-} content (B type > 90%) in the CA ceramic was around 8 wt %. All ceramics were highly crystalline.

Different production techniques caused different macrostructures. Macropores of BCPA and BCPB had a somewhat longitudinal shape, with a length/width aspect ratio between 1.5 and 2. BCPC, BCPD, and CA possessed macropores with a more regular circular shape, with a length/width ratio close to 1. Despite these differences, all ceramics had a macroporous structure, with macropores varying in diameter between 50 and 1000 μm. LM photographs of the ceramics macrostructures prior to implantation are shown in Figure 2.

Microstructures of the ceramics differed significantly, as illustrated by Figure 3. BCPB contained very few micropores and a larger average crystal size in comparison with other investigated ceramics. Macropore walls of the CA ceramic, on the other hand, contained a large amount of micropores and small crystal size as compared with all other ceramics in this study. Crystal size of BCPA was smaller than that of BCPC, while the amount of micropores in BCPD was lower than the amount of micropores in BCPA and BCPC.

Figure 4 exhibits the macroporosities and microporosities of the ceramics prior to implantation, expressed as differential mercury intrusion as a function of pore size. The diameter of most macropores of BCPA, BCPB, and BCPD was around 400 μm, while macropores of CA had a slightly smaller average diameter. Average diameter of BCPC macropores was lower as compared with the other four ceramics. Both image analysis of ESEM pictures and mercury porosimetry measurements indicated that, prior to implantation, macroporosities of all ceramics were similar, 58 ± 5%. Most micropores of all five ceramics had a diameter of around 1 μm. In addition, CA ceramic possessed a high amount of micropores with a very small diameter of around 0.1 μm. Microporosities of BCPA, BCPB, BCPC, BCPD, and CA were circa 17%, 4%, 24%, 10%, and 20%, respectively. Total porosities of BCPA, BCPB, BCPC, BCPD, and CA were 75 ± 5%, 70 ± 5%, 75 ± 5%, 75 ± 5%, and 80 ± 5%, respectively, as measured by mercury porosimetry. Data on macroporosity and microporosity and crystal size can be summarized in specific surface area, which allows for the comparison of these, slightly different, ceramics. Specific surface areas of the five ceramics were as follows: BCPA about 1.0 m^2/g, BCPB about 0.2 m^2/g, BCPC about 1.4 m^2/g, BCPD about 0.8 m^2/g, and CA about 9.7 m^2/g.

Table II gives an overview of material characterization of the five investigated ceramic types.

Intramuscular implantation

Comparison of the materials

There were no surgical complications and all implants were retrieved. At retrieval, all implants were surrounded by well-vascularized muscle tissue. Histology showed no evidence for toxicity of the implants, or signs of an unwanted inflammatory tissue response.

Bone was formed in 9 out of 10 goats in BCPA implants and in 6 out of 10 goats in BCPC implants, while no signs of bone formation were found in BCPB, BCPD, and CA implants (Table II). Figures 5(a–e) show photographs of the histological slides of the five implanted ceramics. In BCPA and BCPC ceramics, bone was formed in the pores of the implants, aligning the ceramic surface, and was never observed on implant peripheries. The newly formed bone was normal in appearance, aligned with osteoblasts, and with osteocytes clearly visible. BCPB, BCPD, and CA ceramics were fully infiltrated by fibrous tissue; however, no signs of bone formation were observed.

An interesting observation from the histological slides was the material degradation behavior. As illustrated in Figure 5(f), in some animals, BCPC exhibited partial fragmentation and delamination of the particles. Interestingly, in these implants, bone was only induced in the part of the implant that was not fragmented (left on the photograph). In addition, in some animals, we observed the loss of structure, probably due to chemical dissolution of the CA ceramic [Fig. 5(g)].

Analyses of the fluorochrome markers confirmed that the bone growth generally started on the surface of the ceramics and continued toward the pore center. For both BCPA [Fig. 5(h)] and BCPC [Fig. 5(i)] ceramic, in some animals, both 6-week oxytetracycline and 8-week xylenol orange marker could be found, which suggested the start of new bone formation before the 6th week of implantation. In other animals, only the xylenol orange marker was found, which emphasizes differences between individual goats, not only in the amount of induced bone but also in the rate of bone formation.

The amount of induced bone largely differed between individual goats, for both BCPA and BCPC. For example, percentage of bone occupying available pore area (area%b/pore) in BCPA varied between 0.3% and 3.5%, with an average of 1.4 ± 1.5%. Area%b/pore in BCPC varied between 0.7% and 4.1%, with an average

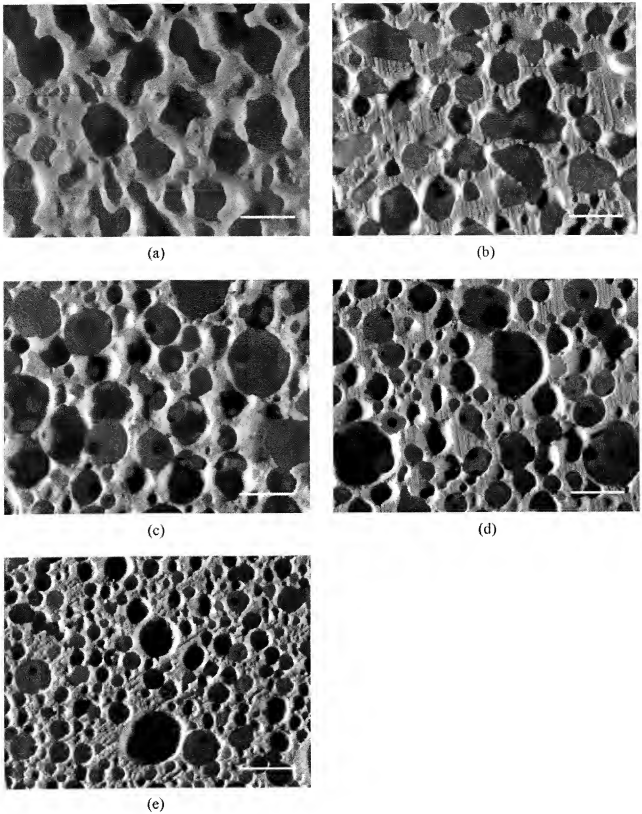
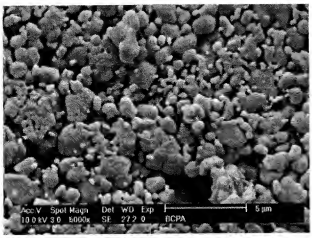
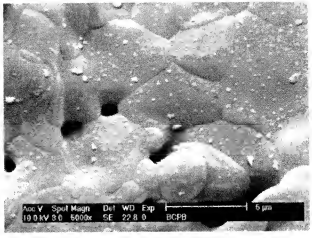


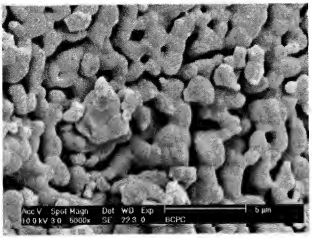
Figure 2. LM photographs (magnification 4×) of BCPA (a), BCPB (b), BCPC (c), BCPD (d), and CA (e) ceramic. Scale bar = 1 mm.



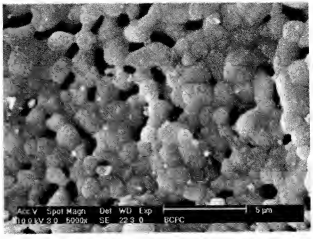
(a)



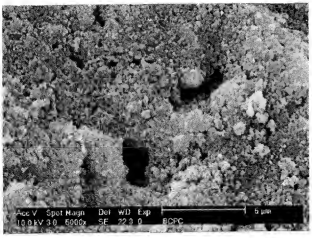
(b)



(c)



(d)



(e)

Figure 3. ESEM photographs (magnification 5000×) of BCPA (a), BCPB (b), BCPC (c), BCPD (d), and CA (e) ceramic.

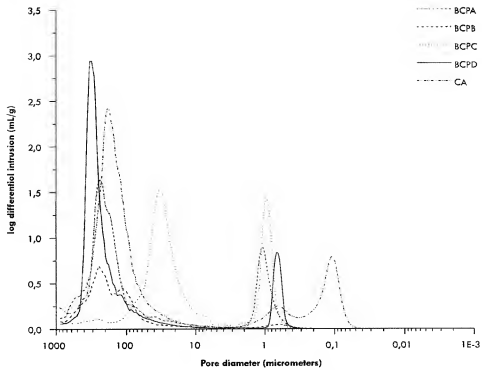


Figure 4. Summary of macroporosities and microporosities and pore size distribution of BCPA, BCPB, BCPC, BCPD, and CA ceramic.

of 1.2 ± 1.3%. Similar to this, area-related parameter, percentage of available scaffold outline in contact with bone (%b.cont.) varied largely between individual goats: %b.cont. in BCPA varied between 1.6% and 8.7%, with an average of 3.9 ± 3.5%; %b.cont. of BCPC varied between 1.9% and 5.6%, with an average of 2.0 ± 2.0%. Histomorphometrical data are summarized in a boxplot (Fig. 6). Although for both measured parameters, BCPA showed a higher value than BCPC, significant difference between the two was only found for %b.cont ($p = 0.011$). Despite large differences in the amount of induced bone between individual goats, an intraanimal consistency was observed. This means that if a relatively large amount of bone was induced by BCPA in goat no.1, in this goat, BCPC induced a large amount of bone as well. And further, if BCPA induced very little bone in goat no.3, in this goat, bone was not induced at all by BCPC. Thus, in all goats, BCPA induced more bone than BCPC; however, there were "more" and "less" inductive goats, and the effect of interanimal variations was visible for both BCPA and BCPC.

Influence of implant size

To investigate the influence of implant size on the amount of induced bone, we compared implants with

TABLE II
Summary of Chemical Compositions, Macroporosities, Specific Surface Areas, and Bone Incidence of the Investigated Ceramics

Implant	Chemical Composition	Macroporosity (vol %)	Microporosity (vol %)	SSA (m^2/g)	Bone Incidence IM	Bone Incidence SC
BCPA	HA/βTCP 70 wt %/30 wt %	58 ± 5	17	1.0	9/10 7/10	NA 0/10
BCPAh	80 wt %/20 wt %					
BCPB	HA/βTCP 80 wt %/20 wt %	58 ± 5	4	0.2	0/10 0/10	NA 0/10
BCPBh						
BCPC	HA/βTCP 70 wt %/30 wt %	58 ± 5	24	1.4	6/10	NA
BCPD	HA/βTCP 70 wt %/30 wt %	58 ± 5	10	0.8	0/10	NA
CA	HA with 8 wt % CO_3^{2-} (type B)	58 ± 5	20	9.7	0/10	NA

the size ø 6.5 × 10 mm^3 (BCPA and BCPB) with the implants with the same diameter, but with half of the length (BCPAh and BCPBh). As already mentioned, BCPA with the full length induced bone in 9 out of 10 animals (Table II). Bone incidence in BCPAh was 7 out of 10 (Table II). In both, large and small implants, the quality of the formed bone was similar. Qualitative analyses of the fluorochrome markers (photographs not shown) showed the presence of both 6-week oxytetracycline and 8-week xylenol orange label in some BCPA implants, while only the xylenol orange label was observed in BCPAh implants, suggesting an earlier start of bone formation in the larger implants. Histomorphometrical data for both measured parameters, percentage of bone occupying available pore area (area%b/pore), and percentage of available scaffold outline in contact with bone (%b.cont.) showed a higher value for BCPA in comparison to BCPAh; however, this difference was only significant ($p = 0.017$) for area%b/pore (Fig. 7).

Neither BCPB nor BCPBh induced bone in any of the animals.

Implantation site dependence

BCPAh and BCPBh implants were implanted both intramuscularly and subcutaneously. As mentioned before, bone incidence in intramuscularly implanted BCPAh was 7 out of 10 (Table II), while no bone was observed in any of the subcutaneously implanted BCPAh ceramics. No bone formation was observed in either intramuscularly or subcutaneously implanted BCPBh ceramics.

Diffusion chamber study

To investigate changes on the surfaces of the ceramics *in vivo*, we subcutaneously implanted BCPA and BCPB ceramics inside diffusion chambers, which allowed the diffusion of macromolecules and ions into the chamber but blocked the invasion of host cells and tissues. Diffusion chambers containing BCPA ceramic and BCPB ceramic, respectively, were implanted for 1, 2, and 4 weeks. Qualitative observation of the two materials after implantation and removal from the chambers showed the macroscopical presence of a glassy layer on the implant surfaces. FTIR spectra of the ceramics after implantation (Fig. 8) exhibited some changes as compared with the spectra before implantation. Already 1 week after implantation of BCPA, new small carbonate bands (C-O in CO_3^{2-}) appeared at 1448 cm^{-1} and 1475 cm^{-1}, indicating the formation of the AB-carbonated apatitic phase. In addition, new, more distinct bands appeared at 1458 cm^{-1} and 1546 cm^{-1}, which correspond to N-H vibrations and a small band at 2960 cm^{-1} corresponding to C-H aliphatic vibrations. The appearance of these bands suggested the presence of organic compounds (proteins, peptides) on the surface of the BCPA ceramic. The above described bands looked even more pronounced in the BCPA FTIR spectrum 2 and 4 weeks after implantation. Changes in the FTIR spectrum of BCPB after implantation were similar to those of BCPA ceramic. However, the newly appeared bands looked somewhat smaller on BCPB than on BCPA spectra. There were no noticeable differences between the XRD patterns of the two ceramics made before and after subcutaneous implantation in diffusion chambers (XRD patterns not shown).

TGA analyses of the two ceramics after implantation were performed in a temperature range 30°C–900°C. The weight loss observed between ±200°C and ±540°C was attributed to organic compounds, while the weight loss between ±540°C and ±900°C was associated with the presence of carbonate, from both CA precipitate and possibly trapped body fluid in the pores. Although not calculated in the present experiment, it should be noted that some water loss could also have occurred in the latter temperature range because of nonstoichiometry and the possible presence of HPO_4^{2-} groups, but it is conceivable that this amount of water loss did not significantly differ between the two investigated ceramics. TGA analysis of the BCPA and BCPB ceramics prior to implantation proved the absence of both organic compounds and carbonate in the initial ceramics. Quantitative data of the TGA analyses are summarized in Figure 9. At all three time points, that is, 1, 2, and 4 weeks of implantation, a significantly higher (in all cases, $p < 0.01$) amount of organic compounds was found on BCPA ceramic surface as compared with the BCPB surface. The amount of carbonate on the surfaces of the ceramics was much smaller than the amount of organic compounds, but also in this case, the carbonate amount on the BCPA surface was significantly higher ($p = 0.015$ for 1 week, $p = 0.009$ for 2 weeks, and $p = 0.019$ for 4 weeks of implantation) than that on BCPB surface, for all time points. No significant differences were observed in the amounts of organic compounds and carbonate between the three time points, for either of the investigated ceramics.

DISCUSSION

Osteoinduction by CaP-containing biomaterials in various forms, as well as by materials that initially do not contain CaPs, has been shown in various reports in the last decade.[12–28,33–35] However, so far, little is known about the exact mechanism of the phenome-

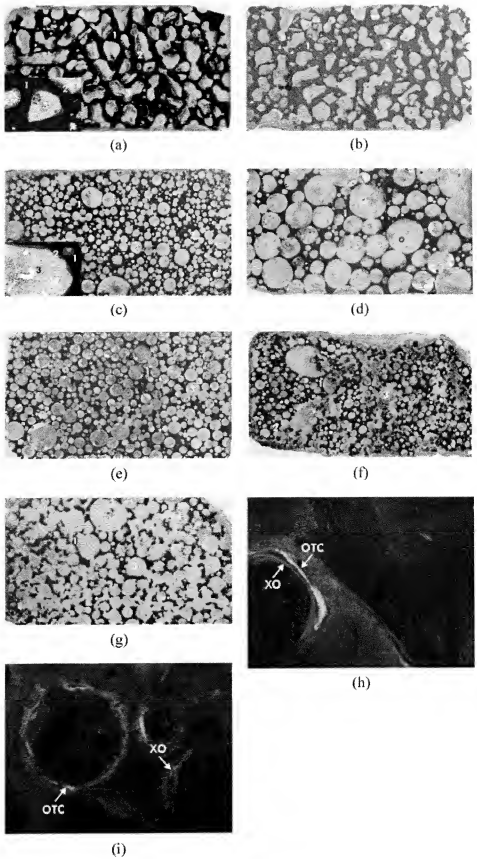


Figure 5.

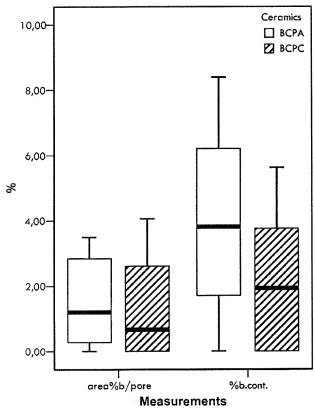


Figure 6. Histomorphometrical results: boxplots (mean and interquartile values) of area%b/pore and %b.cont. of intramuscularly implanted BCPA and BCPC ceramics. For both, bone area-related parameter (area%b/pore) and bone contact parameter (%b.cont.) BCPA performed better than BCPC, but the difference was only significant ($p = 0.011$) for %b.cont.

Figure 5. Digital photograph of histological slides of intramuscularly implanted BCPA (a) (inset = LM photograph magnification 10×), BCPB (b), BCPC (c) (inset = LM photograph magnification 10×), BCPD (d), CA (e), BCPC (f), CA (g), and fluorochrome markers of BCPA (h) and BCPC (i). 1 indicates ceramic; 2, bone; 3, fibrous tissue; OTC, oxytetracycline; and XO, xylenol orange. The induced bone in BCPA and BCPC (a and c, respectively) is formed in the pores of the implants, aligning their surface. The bone is normal in appearance, aligned with osteoblasts and with osteocytes and osteoid clearly visible (insets of a and c). BCPB, BCPD, and CA ceramics (b, d, and e, respectively) are extensively filled with fibrous tissue, but no signs of bone formation are observed. In some animals, BCPC ceramic was broken and mechanical degradation was observed. Note bone formation only in the nonfragmented part of the implant (f). In some animals, chemical dissolution of CA ceramic was observed (g). Presence of both oxytetracycline and xylenol orange marker shows that the bone formation in both BCPA and BCPC (h and i, respectively) had started before the sixth week of implantation. [Color Figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

non underlying osteoinduction. The results of this study add to the knowledge of parameters that influence material osteoinductive properties.

The first goal of this study was to further investigate the influence of physicochemical and structural properties of a material on its osteoinductive properties. We compared five CaP ceramics, four of which were BCPs. Although produced from powders with a similar chemical composition, these four ceramics had quite different final properties. As previously described, BCPA and BCPB, with the same chemical composition and macrostructure, differed only in their microstructures because of differences in the temperatures they were sintered at[27]: BCPA had a highly porous microstructure, while hardly any micropores could be found in the macropore walls of BCPB. A consequence of the different microstructures was that the specific surface area of BCPA was about 5 times higher than that of BCPB. After implantation, BCPA induced bone in almost all animals. In contrast, no bone was found in any of the implanted BCPB ceramics. In agreement with previous studies, these findings suggest that below a certain level of specific surface

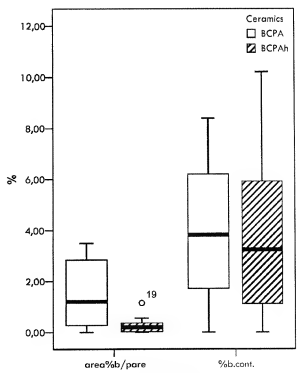


Figure 7. Histomorphometrical results: boxplots (mean and interquartile values) of area%b/pore and %b. between intramuscularly implanted BCPA and BCPAh ceramics. (o) = outlier. For both, bone area-related parameter (area%b/pore) and bone contact parameter (%b.cont.) BCPA implant (ø 6.5 × 10 mm^3) showed more bone than a similar, but smaller (ø 6.5 × 5 mm^3) implant (BCPAh), but the difference was only significant ($p = 0.017$) for area%b/pore.

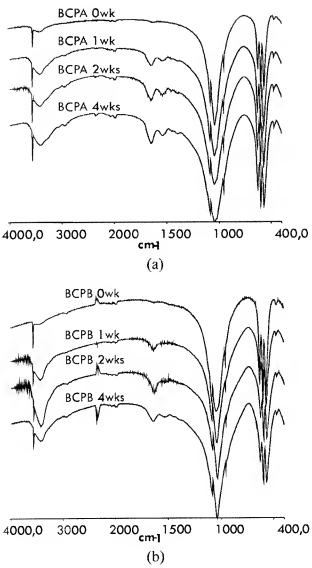


Figure 8. FTIR spectra of BCPA (a) and BCPB (b) implanted subcutaneously inside diffusion chambers for 1, 2, and 4 weeks.

area, bone will not be induced. Therefore, a "high" specific surface area seems to be of essential importance for osteoinduction by biomaterials.

BCPC and BCPD were produced from the same powder, but using different techniques. They had the same chemical composition, and similar macroporosities, but different microporosities. Although both are microporous, the amount of micropores found in BCPC was higher as compared with BCPD. In addition, crystal size of BCPC was smaller than that of BCPD. Similar to the BCPA and BCPB ceramics, a consequence of more micropores and smaller crystal size was an around two times higher specific surface area of BCPC than that of BCPD. After implantation, BCPC induced bone in 7 out of 10 animals, while no bone was observed in any of the BCPD implants. Similar to the comparison of BCPA and BCPB, also in this case, an increased specific surface area positively influenced material's osteoinductive potential.

The comparison of the two osteoinductive ceramics in this study, namely, BCPA and BCPC, is slightly more complicated. BCPC contained 10% more β-TCP than did BCPA. In addition, the specific surface area of BCPC was around 1.5 times higher than that of BCPA. Both the higher amount of the more soluble component β-TCP and a higher specific surface area obviously increase the surface reactivity of a material, which should, as previously proposed,[20] improve its osteoinductive properties. This was, however, not the case in the present study. Besides a lower bone incidence in BCPC, the amount of bone induced in BCPC was lower than the amount found in BCPA. Further differences between these two ceramics, which could possibly explain these unexpected results, are found in their macrostructures. Although both are highly macroporous, most macropores of BCPA had a diameter of around 400 μm, while most macropores of BCPC had an average diameter smaller than 100 μm. As recently reviewed by Karageorgiou and Kaplan,[49] based on early studies, the minimum requirement for pore size is considered to be ±100 μm because of cell size, migration requirements, and nutrient transport.[50] However, pore sizes higher than 300 μm are recommended because of the formation of capillaries. Although these recommendations were based on processes of bone formation that take place orthotopically, it is conceivable that they are also applicable for the process of osteoinduction. It is thus possible, that although the higher surface reactivity of BCPC was preferable for osteoinduction according to the earlier hypothesized mechanism,[20] BCPA's larger pores were responsible for better nutrient and cell distribution and capillary formation throughout the implant. Nutrient and cell distribution and consequent capillary formation should precede the process of ectopic bone formation and if they are insufficient, this process may be delayed or decreased. Furthermore, BCPC ceramic, as is shown in the results paragraph, partially fragmented after implantation in some of the animals. This resulted in the formation of loose particles and debris, and in the loss of macroporous structure. Investigation of histological slides showed that in the fragmented parts of the implant, no bone could be found. This observation could be explained by the fact that in order to facilitate growth of the induced bone, a mechanically stable surface is needed. Micromotion caused by debris and loose particles may negatively influence the bone growth facilitation.

The CA ceramic had a specific surface area, which was significantly higher that that of the other ceramics used in this study. Furthermore, its chemical composition, which closely resembles that of bone mineral, was different from that of the four BCPs, which makes

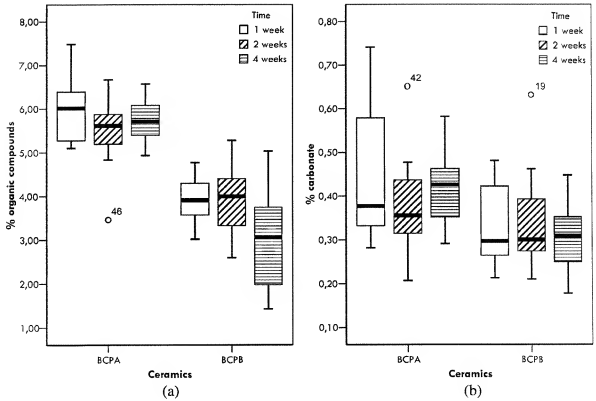


Figure 9. TGA analysis: boxplots (mean and interquartile values) of %organic compounds (a) and carbonate (b) in subcutaneously implanted BCPA and BCPB ceramic inside diffusion chambers for 1, 2, and 4 weeks. (o) = outliers. The amount of both organic compounds and carbonate was significantly higher in BCPA than in BCPB at all implantation time points. No significant difference between the time points was observed for neither of the measured compounds.

the comparison more challenging. Although we retrieved all implanted materials, histological slides showed a significant dissolution of the CA ceramic in some animals. This observation suggests that there is a limit in the increase of the specific surface area of the material that positively influences its osteoinductive properties. In the case of CA ceramic, chemical composition and a very high specific surface area might be the reasons for the extensive dissolution of the material before the bone formation has started. Histological observations did not indicate different cellular activity on the surface of different materials, which suggests that the observed dissolution of the CA ceramic was spontaneous chemical dissolution. Obviously, the rate of dissolution of calcium and phosphate ions was very high, causing the loss of macroporous structure which is necessary for reaching the supersaturation of these ions, and consequent reprecipitation of the biological apatite. Thus, although the induction of differentiation toward osteoblasts might have taken place, due to the high dissolution of the surface, bone formation could not be facilitated.

In short, the results from the part of the study in which the influence of physicochemical properties on osteoinduction was investigated showed that on the one hand, bone is not induced below a certain low level of specific surface area and on the other hand, bone formation, although possibly initiated, does not take place above a certain high level of specific surface area because of a fast resorption. These findings suggest that for each material type there is an optimal specific surface area which leads to maximal osteoinduction, if any. In addition, results of this study emphasized previous observations that the right macrostructure (i.e., macropore size, interconnectivity), which leads to required nutrient and cell supply and vascularization, are of great importance in the process of osteoinduction by biomaterials. Finally, a mechanically stable surface is needed for facilitation of initiated bone formation.

It should be noted that, in addition to the microstructure that is responsible for differences of the specific surface area of the investigated materials, there are other surface characteristics that might influence osteoinductive potential of the material as well. For example, as reviewed by Nancollas et al.,[51] various, dependent surface properties, such as crystal morphology, surface charge, and energy, might all alter the adsorption and the nucleation characteristics of the surface. However, the fact that all materials used in

the present study were macroporous and microporous makes the characterization of their surface at the level of charge and energy highly complex. On the other hand, it is well known that materials that are not porous lack osteoinductive potential. Future research should therefore focus on developing techniques that allow for a more detailed surface characterization of highly porous materials.

Results of the implant-size investigation showed that bone incidence in BCPA implants with the size ø 6.5×10 mm^3 was higher than in similar implants with half the length (ø 6.5×5 mm^3). Histomorphometrical measurements further confirmed that the relative amount of bone in available pore area was relatively higher in the bigger than in the smaller implant. The observed implant size influence could be explained by the fact that, in comparison to smaller implants, larger implants have a larger periphery surface, which allows for more migration of cells and nutrients inside the implant. Furthermore, there is possibly more micromotion in the smaller implants as compared with the larger ones, which could negatively influence the process of attachment, proliferation, and differentiation of the cells in the centre of the implant. This implant-size dependence might be, in addition to the well-known interspecies genetic and metabolic differences, one of the reasons that explains the lack of osteoinduction by biomaterials in rats and mice, in which obviously only small implants can be inserted.

Comparison of implantation sites showed that intramuscularly implanted BCPA induced bone, while no bone was found in any of the subcutaneously implanted BCPA ceramics, which is possibly due to a better vasularization intramuscularly. These results contrast the findings of Gosain et al.,[19] in which authors compared intramuscular and subcutaneous osteoinduction in sheep after 1 year of implantation, and could not find any significant differences between the two sites. Although a similar type of comparison is made, our study in goats and Gosain et al.'s study in sheep differed in osteoinductive materials used, implantation time, and, most important, animal model. Our observation that subcutis was a less inductive implantation site than muscle was, however, in accordance with the finding of Yang et al.[43] Although this study lacked histomorphometrical data, the authors observed a delayed osteoinduction in subcutaneously implanted materials as compared with intramuscularly implanted materials in both pigs and dogs. The observations of the latter study suggest that there might be a difference in "osteoinductive potential" between subcutis and muscle, which is conceivable as it is generally accepted that there is more vascularization intramuscularly than subcutaneously. However, the difference between the two sites might be visible only during the early time of implantation. It is plausible that, after a certain implantation time, the ectopically induced bone stops growing and starts resorbing as it lacks natural mechanical stimulation. The process of bone resorption occurs earlier intramuscularly than subcutaneously, as the bone formation intramuscularly takes place earlier as well. It is therefore possible that, after 1 year of implantation as in Gosain et al.'s study, difference in the amount of bone between subcutaneously and intramuscularly implanted materials is not visible anymore, while in our 3-month study, this difference was clearly observed.

As previously mentioned, osteoinduction by biomaterials shows large interspecies differences. The bone is hardly ever induced in rats and mice, [12,40–43] is rarely found in rabbits, and is found very frequently in large mammals (i.e., goats, sheep, dogs) and nonhuman primates such as baboons.[13,19,26,28,43,52,53] However, there is also a difference in osteoinductive potentials between different large animals. The same material implanted intramuscularly in goats and dogs induced more bone in dogs.[43] Similarly, a material implanted in dogs and baboons induced more bone in baboons.[13] To our knowledge, direct comparisons between sheep and goats in the same study have not been made. It is hard to make comparisons between our study in goats and the studies in sheep, as, in addition to the difference in the materials used to study osteoinduction, the implantation time of Gosain et al.'s study was 1 year and Le Nihouannen et al.'s[28] study was 6 months while implantation time of our study was 3 months. Besides, in the study of Le Nihouannen et al.[28] the volume of implanted ceramic was around 6 times higher than the volume of the present study, and this, as showed above, might be of importance for the amount of induced bone. When comparing studies on osteoinduction by biomaterials, it is therefore important to take into account that, in addition to the animal model and material used, implantation time, implantation site, and the amount of implanted material may be of great importance for the amount of induced bone.

Implantation of osteoinductive and nonosteoinductive ceramic inside diffusion chambers was meant to investigate changes on the material surface, without interference with cells and tissues. Implantation periods of 1, 2, and 4 weeks were chosen because, in the chosen goat intramuscular model, ectopic bone formation starts before the sixth week of implantation, as is shown by the fluorochrome markers. The results of FTIR and TGA analyses on the materials explanted and removed from the diffusion chambers support the hypothesis that in the process of osteoinduction, surface reactivity plays a role. It is proposed that on the surface of the material, a dissolution-reprecipitation process takes place, which is accompanied by coprecipitation of relevant endogeneous factors such as cytokines or proteins.[20] On a material with a higher specific surface area, this process of induction is faster

and more prominent. Although the environment in a diffusion chamber is not fully identical to the one in the muscle, the results from the simplified model of the present study indeed show that during the implantation more carbonate and more organic compounds were found on the surface of the osteoinductive BCPA ceramic as compared with the nonosteoinductive BCPB ceramic. Whether this process is essential for the osteoinductivity of a material needs to be further proven. However, these results give a direct relationship between specific surface area and the material interaction with its *in vivo* environment. The next step in the finding the mechanism of osteoinduction would be to investigate which organic compounds are present on the material surface, and what is their role in the induction of ectopic bone formation.

CONCLUSION

The results of the present study emphasized the importance of physicochemical and structural parameters for osteoinductive properties of biomaterials. The presence of micropores, by which the specific surface area of a material is increased, is essential for the material's osteoinductivity. The increased specific surface area leads to more surface reactivity in terms of dissolution and reprecipitation of a biological apatite layer, which possibly coprecipitates relevant endogenous proteins, and finally triggers the undifferentiated cells to start differentiating toward the osteogenic lineage. However, if the surface area is too high, or material is too resorbable because of its chemical composition, the implant might degrade and lose its shape. In that case, ectopic bone formation does not occur, as a relatively stable surface is needed to facilitate new bone growth.

This study also showed that the decrease of the implant size significantly decreases the relative amount of induced bone by a material. In addition, in the model used in this study, the same material induced bone intramuscularly but not subcutaneously, which suggests the importance of vascularization in the process of osteoinduction by biomaterials.

References

1. Bridges JB, Pritchard JJ. Bone and cartilage induction in the rabbit. J Anat 1958;92(1):28–38.
2. Urist MR, McLean FC. Osteogenetic potency and new-bone formation by induction in transplants to the anterior chamber of the eye. *J Bone Joint Surg Am* 1952;34-A(2):443–476.
3. Lacroix P. Recent investigation on the growth of bone. *Nature* 1945;156:576.
4. Bertelsen A. Experimental investigations into post-foetal osteogenesis. *Acta Orthop Scand* 1944;15:139–181.
5. Urist MR, Silverman BF, Buring K, Dubuc FL, Rosenberg JM. The bone induction principle. Clin Orthop 1967;53:243–283.
6. Friedenstein AY. Induction of bone tissue by transitional epithelium. *Clin Orthop Relat Res* 1968;59:21–37.
7. Reddi AH, Huggins C. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats Proc Natl Acad Sci U S A 1972;69(6):1601–1605.
8. Reddi AH. Cell biology and biochemistry of endochondral bone development. *Coll Relat Res* 1981;1(2):209–226.
9. Urist MR. Bone: formation by autoinduction. *Science* 1965;150(698):893–899.
10. Urist MR, DeLange RJ, Finerman GA. Bone cell differentiation and growth factors. *Science* 1983;220(4598):680–686.
11. Winter GD, Simpson BJ. Heterotopic bone formed in a synthetic sponge in the skin of young pigs. Nature 1969;223(201):88–90.
12. Klein C, de Groot K, Chen W, Li Y, Zhang X. Osseous substance formation induced in porous calcium phosphate ceramics in soft tissues. Biomaterials 1994;15(1):31–34.
13. Ripamonti U. Osteoinduction in porous hydroxyapatite implanted in heterotopic sites of different animal models. *Biomaterials* 1996;17(1):31–35.
14. Yamasaki H. Heterotopic bone formation around porous hydroxyapatite ceramics in the subcutis of dogs. *Jpn J Oral Biol* 1990;32:190–192.
15. Yamasaki H, Sakai H. Osteogenic response to porous hydroxyapatite ceramics under the skin of dogs. Biomaterials 1992; 13(5):308–312.
16. Zhang X. A study of porous block HA ceramics and its osteogenesis. In: Ravaglioli A, Krajewski A, editors. Bioceramics and the Human Body. Amsterdam, The Netherlands: Elsevier Science; 1991, p 692–703.
17. Ripamonti U. The morphogenesis of bone in replicas of porous hydroxyapatite obtained from conversion of calcium carbonate exoskeletons of coral. *J Bone Joint Surg Am* 1991;73(5):692–703.
18. Vargervik K. Critical sites for new bone formation. In: Habal MB, Reddi AH, editors. Bone Grafts and Bone Substitutes. Philadelphia: WB Saunders; 1992, p 112–120.
19. Gosain AK, Song L, Riordan P, Amarante MT, Nagy PG, Wilson CR, Toth JM, Ricci JL. A 1-year study of osteoinduction in hydroxyapatite-derived biomaterials in an adult sheep model: part I. Plast Reconstr Surg 2002;109(2):619–630.
20. Habibovic P, Yuan H, van der Valk CM, Meijer G, van Blitterswijk CA, de Groot K. 3D microenvironment as essential element for osteoinduction by biomaterials. Biomaterials 2005; 26(17):3565–3575.
21. Habibovic P, Li J, van der Valk CM, Meijer G, Layrolle P, van Blitterswijk CA, de Groot K. Biological performance of uncoated and octacalcium phosphate-coated Ti6Al4V. Biomaterials 2005;26(1):23–36.
22. Yuan H, Yang Z, Zou P, Li Y, Zhang Z. An investigation of the osteoinduction of synthetic porous phase-pure hydroxyapatite ceramic. Biomed Eng Appl Bas Com 1997;9:274–278.
23. Yuan H, Yang Z, Li Y, Zhang Z, de Bruijn JD, de Groot K. Osteoinduction by calcium phosphate biomaterials. J Mater Sci Mater Med 1998;9:723–726.
24. Yuan H, Kurashina K, de Bruijn JD, Li Y, de Groot K, Zhang X. A preliminary study on osteoinduction of two kinds of calcium phosphate ceramics. Biomaterials 1999;20(19):1799–1806.
25. Yuan H, de Bruijn JD, Li Y, Feng Z, Yang K, de Groot K, Zhang X. Bone formation induced by calcium phosphate ceramics in soft tissue of dogs: a comparative study between alpha-TCP and beta-TCP. J Mater Sci Mater Med 2001;12:7–13.
26. Yuan H, van den Doel M, Li SH, van Blitterswijk CA, de Groot K, de Bruijn JD. A comparison of the osteoinductive potential of two calcium phosphate ceramics implanted intramuscularly in goats. J Mater Sci Mater Med 2002;13:1271–1275.

27. Habibovic P, Yuan H, van den Doel M, Sees TM, van Blitterswijk CA, de Groot K. Relevance of osteoinductive biomaterials in a critical-sized orthotopic defect. *J Orthop Res.* Forthcoming.
28. Le Nihouannen D, Daculsi G, Saffarzadeh A, Gauthier O, Delplace S, Pilet P, Layrolle P. Ectopic bone formation by microporous calcium phosphate ceramic particles in sheep muscles. Bone 2005;36(6):1086–1093.
29. Yuan H, Li Y, de Bruijn JD, de Groot K, Zhang X. Tissue responses of calcium phosphate cement: a study in dogs. Biomaterials 2000;21(12):1283–1290.
30. Barrere F, van der Valk CM, Dalmeijer RA, Meijer G, van Blitterswijk CA, de Groot K, Layrolle P. Osteogenecity of octacalcium phosphate coatings applied on porous metal implants. J Biomed Mater Res 2003;66A(4):779–788.
31. Habibovic P, van der Valk CM, Meijer G, van Blitterswijk CA, de Groot K. Influence of octacalcium phosphate coating on osteoinductive properties of biomaterials. J Mater Sci Mater Med 2004;15(4):373–380.
32. Yuan H, de Bruijn JD, Zhang X, van Blitterswijk CA, de Groot K. Bone induction by porous glass ceramic made from Bioglass (45S5). J Biomed Mater Res 2001;58(3):270–276.
33. Yuan H, de Bruijn JD, Zhang X, van Blitterswijk CA, de Groot K. Osteoinduction by Porous Alumina Ceramic. London: European Society for Biomaterials; 2001. p 209.
34. Fujibayashi S, Neo M, Kim HM, Kokubo T, Nakamura T. Osteoinduction of porous bioactive titanium metal. Biomaterials 2004;25(3):443–450.
35. Takemoto FSM, Matsushita T, Suzuki J, Kokubo T, Nakamura T. Osteoinductive Ability of Porous Titanium Implants Following Three Types of Surface Treatment. Washington, DC: Orthopaedic Research Society; 2005. p Poster No: 0992.
36. Ripamonti U, Crooks J, Kirkbride AN. Sintered porous hydroxyapatites with intrinsic osteoinductive activity: geometric induction of bone formation. S Afr J Sci 1999;95:335–343.
37. Reddi AH. Regulation of cartilage and bone differentiation by bone morphogenetic proteins. *Curr Opin Cell Biol* 1992;4(5):850–855.
38. Reddi AH. Bone and cartilage differentiation. *Curr Opin Genet Dev* 1994;4(5):737–744.
39. Wozney JM. The bone morphogenetic protein family and osteogenesis. *Mol Reprod Dev* 1992;32(2):160–167.
40. Goshima J, Goldberg VM, Caplan AI. The osteogenic potential of culture-expanded rat marrow mesenchymal cells assayed in vivo in calcium phosphate ceramic blocks. Clin Orthop Relat Res 1991;262:298–311.
41. Ohgushi H, Dohi Y, Tamai S, Tabata S. Osteogenic differentiation of marrow stromal stem cells in porous hydroxyapatite ceramics. J Biomed Mater Res 1993;27(11):1401–1407.
42. Ohgushi H, Goldberg VM, Caplan AI. Heterotopic osteogenesis in porous ceramics induced by marrow cells. J Orthop Res 1989;7(4):568–578.
43. Yang Z, Yuan H, Tong W, Zou P, Chen W, Zhang X. Osteogenesis in extraskeletally implanted porous calcium phosphate ceramics: variability among different kinds of animals. Biomaterials 1996;17(22):2131–2137.
44. Ripamonti U. Smart biomaterials with intrinsic osteoinductivity: geometric control of bone differentiation. In: Davies J, editor. Bone Engineering. Toronto, Canada: em squared Corporation; 2000, p 215–222.
45. Heughebaert M, LeGeros RZ, Gineste M, Guilhem A, Bonel G. Physicochemical characterization of deposits associated with HA ceramics implanted in nonosseous sites. *J Biomed Mater Res* 1988;22(3 Suppl):257–268.
46. Kruyt MC, de Bruijn JD, Wilson CE, Oner FC, van Blitterswijk CA, Verbout AJ, Dhert WJ. Viable osteogenic cells are obligatory for tissue-engineered ectopic bone formation in goats. Tissue Eng 2003;9(2):327–336.
47. Kruyt MC, Dhert WJ, Yuan H, Wilson CE, van Blitterswijk CA, Verbout AJ, de Bruijn JD. Bone tissue engineering in a critical size defect compared to ectopic implantations in the goat. J Orthop Res 2004;22(3):544–551.
48. Mendenhall W. Nonparametric Statistics: Introduction to Probability and Statistics. Belmont, UK: Duxbury Press; 1971.
49. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials 2005;26(27):5474–5491.
50. Hulbert SF, Young FA, Mathews RS, Klawitter JJ, Talbert CD, Stelling FH. Potential of ceramic materials as permanently implantable skeletal prostheses. J Biomed Mater Res 1970;4(3):433–456.
51. Nancollas GH, Wu W, Tang R. The control of mineralization on natural implant surfaces. In: Li P, Calvert P, Kokubo T, Levy R, Scheid CH, editors. Materials Research Society Symposium Proceedings. Boston: Library of Congress Cataloging-in-Publishing Data; 1999.
52. Ripamonti U, van den Heever B, van Wyk J. Expression of the osteogenic phenotype in porous hydroxyapatite implanted extraskeletally in baboons. Matrix 1993;13(6):491–502.
53. Yang Z, Yuan H, Zou P, Tong W, Qu S, Zhang X. Osteogenic response to extraskeletally implanted synthetic porous calcium phosphate ceramics: an early stage histomorphological study in dogs. J Mater Sci Mater Med 1997;8:697–701.